



Published in final edited form as:

Mol Psychiatry. 2010 June ; 15(6): 657–668. doi:10.1038/mp.2009.97.

Ovarian Steroids Decrease DNA Fragmentation In Serotonin Neurons of Non-injured Rhesus Macaques

Fernanda B. Lima^a and Cynthia L. Bethea^{a,b}

^aDivision of Reproductive Sciences Oregon National Primate Research Center Beaverton, Oregon, 97006, USA

^bDivision of Neuroscience Oregon National Primate Research Center Beaverton, Oregon, 97006, USA

Abstract

We previously found that ovarian steroids promote neuroprotection in serotonin neurons by decreasing the expression of pro-apoptotic genes and proteins in the dorsal raphe nucleus of rhesus macaques, even in the absence of overt injury. In this study, we questioned whether these actions would lead to a reduction in DNA fragmentation in serotonin neurons. Ovariectomized (OVX) rhesus monkeys received Silastic implants that were empty (placebo) or containing estradiol (E), progesterone (P) or estradiol plus progesterone (E+P) for one month. Eight levels of the dorsal raphe nucleus in a rostral to caudal direction were immunostained with TUNEL (terminal deoxynucleotidyl transferase nick end labeling). Two staining patterns were observed, which are referred to as type I, with complete dark staining of the nucleus, and type II, with peripheral staining in the perinuclear area. A montage of the dorsal raphe was created at each level with a Marianas Stereology Microscope and Slidebook 4.2 and TUNEL positive cells were counted. In direct comparison with OVX animals, P treatment and E+P treatment significantly reduced the total number of TUNEL positive cells (Mann Whitney test, both treatments $p=0.04$) and E+P treatment reduced the number of TUNEL positive cells/cubic millimeter (Mann Whitney test, $p=0.04$). Double immunocytochemistry for TUNEL and TPH indicated that DNA fragmentation was prominent in serotonin neurons. These data suggest that in the absence of ovarian steroids, a cascade of gene and protein expression leads to an increase in DNA fragmentation in serotonin neurons. Conversely, ovarian steroids have a neuroprotective role in the non-injured brain and prevent DNA fragmentation and cell death in serotonin neurons of nonhuman primates.

Keywords

Apoptosis; necrosis; TUNEL; estrogen; progesterone; serotonin; dorsal raphe nucleus

Users may view, print, copy, download and text and data- mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use: http://www.nature.com/authors/editorial_policies/license.html#terms

Corresponding author: Cynthia L. Bethea Division of Reproductive Sciences, Oregon National Primate Research Center 505 NW 185th Avenue Beaverton, OR 97006, USA. Fax: +1 503 690 5384. betheac@ohsu.edu.

Introduction

In women, premature ovarian failure or menopause, is characterized by a significant decline in circulating levels of the ovarian steroids, estrogen and progesterone, and a large body of literature indicates that estrogen and progesterone are neuroprotective (1-3). By the early 1990's, studies of the degeneration, neuroprotection and regeneration of CNS neurons departed from descriptions of neurons as either dead or alive and the notion of neuronal health as a dynamic equilibrium that spans a spectrum between resilient and vulnerable neuronal states came to be accepted (4). Thus, at any given time, neurons may be very healthy and functioning well including optimal DNA repair, or somewhere on a slippery slope where they are unhealthy and falling farther and farther behind in DNA repair but not dead. The concept of "neuroendangerment" has been proposed for these unhealthy neurons (5). They are vulnerable and additional stresses could kill them. However, up to the point of no return, recovery may be possible. Many factors could render a neuron vulnerable. Endogenous factors such as imbalances in homeostasis, hormones, growth factors, cytokine milieu, genetics and ageing probably play important roles. External factors such as stress, diet, exercise, disease and medications are transduced into live or die signals for neurons in ways that are not fully understood. However, systemic infection and cytokines clearly contribute to neuroendangerment (6).

Neurodegeneration is usually thought of in the context of severe deficits in motor or cognitive function whereas depression and mood disorders have traditionally been viewed as neurochemical deficits. However recently, it has been suggested that even the psychopathologies may involve functional degeneration of critical central neural systems and many are thought to have a serotonergic etiology (7, 8). Moreover, evidence is increasing that depression is accompanied by marked changes in the number or size of neurons and glia in discrete brain regions (5). Stressful life events are one of the major predisposing risk factors for developing depression, and stress-induced atrophy occurs in hippocampal neurons of rodents (9).

A prominent feature of neuroprotection is the inhibition of apoptosis. Apoptosis, also called programmed cell death, is a homeostatic process under genetic control that has been evolutionarily conserved to balance cell replication and optimize cellular organization. It is characterized by condensation of chromatin, cell shrinkage, nuclear fragmentation and formation of apoptotic bodies, and the resulting cells are later ingested by phagocytes (10-12). Apoptosis may proceed through activation of caspases (caspase-dependent pathways) or through translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus (caspase-independent pathway) (13)

In tree shrews, stress increased the incidence of apoptosis in the temporal cortex. Moreover, antidepressant treatment reduced apoptotic neurons to control unstressed levels (14). Nonetheless, examples of serotonin cell death are lacking. The midbrain has not received the attention of the forebrain areas, yet it is the location of the serotonin and norepinephrine cell bodies, and these cells control many forebrain functions associated with stress responsivity (15). Thus, any loss or degeneration of serotonin neurons could have profound ramifications.

We have begun to question whether the steroid hormones, E and P, play a role in serotonin neuron resilience or vulnerability.

Our studies indicate that serotonin neurons contain estrogen receptor beta (ER β) (16) and progesterone receptors (PR) (17). Estrogen and progesterone regulate gene and protein expression in serotonin neurons in a manner that would increase serotonin production, increase serotonin turnover, increase serotonin neural firing and decrease serotonin degradation (18-20).

Recently, using Affymetrix array analysis on RNA extracted from the dorsal raphe region or from laser captured serotonin neurons, we found that several genes involved in neurotoxicity such as kynurenin mono-oxygenase (KMO), or programmed cell death such as c-jun n-terminal kinase (JNK), apoptosis inducing factor (AIF), apoptotic peptidase activating factor (APAF), and DIABLO were decreased by one month of hormone therapy (21, 22). In a further study of protein expression in a small block of tissue containing the serotonin neurons of the dorsal raphe nucleus, we showed that estrogen plus progesterone administration decreased the expression of JNK1 and AIF, as well as decreased the translocation of AIF from the mitochondria to the nucleus (23). These studies suggested that in the absence of injury, ovarian steroids may decrease apoptosis through a caspase-independent pathway in serotonin neurons.

It is important to note that our model of hormone therapy in primates after surgical menopause does not involve a gross insult to the brain. So, although the neuroprotective effect of hormone therapy has been extensively studied in models of injury to the brain, the neuroprotective actions of hormone therapy in a non-injured environment are not as well understood. However, there is a continuum of lesser insults to the CNS over the course of life such as stress, illness and psychological trauma. Thus, we questioned the potential of ovarian steroids to increase the resilience of serotonin neurons in the absence of injury.

Several methods are available to detect apoptosis or necrosis. In this study we used the TUNEL assay to determine the effect of hormone therapy on DNA fragmentation in the dorsal raphe nucleus and to determine if the fragmentation was occurring in serotonin neurons.

Materials and Methods

The Oregon National Primate Research Center Institutional Animal Care and Use Committee approved this study.

Animals and treatments

We utilize a non-human primate model of surgical menopause with hormone replacement. Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized following their use in other protocols by the surgical personnel of ONPRC according to accepted veterinary surgical protocol. Recently, the time between ovariectomy and initiation of hormone therapy has become a critical issue. Ideally, all the monkeys in a study should be ovariectomized for the same length of time. However, due to cost, we acquired animals that were

ovariectomized in other programs and then released into the available pool for terminal studies (1/7 cost of an intact female). We aimed to use monkeys that were ovariectomized within a reasonably short time frame, but long enough to rest and recover from previous protocols. Thus, our animals were assigned between 3 and 8 months after ovariectomy and the average length of time equaled 5.5 months. All animals were born in China and were aged between 7 and 14 years by dental exam. The average age of the animals for which the age was known equaled 8.8 years. They weighed between 4 and 8 kg, and were in good health. Steroid hormone treatments were initiated 3-8 months after ovariectomy. Animals were either treated with placebo (Ovx control group; n=5), or treated with estradiol for 28 days (E group; n=5), or treated with estradiol for 28 days and then supplemented with progesterone for the final 14 of the 28 days (E+P group; n=5), or treated with placebo for 14 days and then treated with progesterone for 14 days (P group; n=5). All twenty animals were processed for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL).

The ovariectomized control monkeys received empty Silastic capsules (s.c.). The E-treated monkeys were implanted with a 4.5-cm E-filled Silastic capsules (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI) filled with crystalline estradiol [1,3,5(10)-estratrien-3,17- β -diol; Steraloids, Wilton, NH]. The E+P-treated monkeys received an E-filled capsule and 14 days later, received one 6-cm capsule filled with crystalline P (4-pregnen-3,20 dione; Steraloids). The P-treated monkeys received a placebo capsule and then one 6-cm capsule filled with crystalline P on day 14. All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA). The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

Tissue Preparation for immunohistochemistry

Following euthanasia, the left ventricle of the heart was cannulated and the head of each animal was perfused with 1 liter of saline followed by 7 liters of 4% paraformaldehyde in 3.8% borate, pH 9.5 (both solutions made with DEPC-treated water [0.1% diethyl pyrocarbonate] to minimize RNase contamination). The brains were removed and dissected. Tissue blocks were post-fixed in 4% paraformaldehyde for 3 h, then transferred to 0.02M potassium phosphate-buffered saline (KPBS) containing 10%, followed by 20% glycerol and 2% dimethyl sulfoxide at 4°C for 3 days to cryoprotect the tissue. After infiltration, the block was frozen in isopentane cooled to -55°C, and stored at -80°C until sectioning. Sections (25 μ m) were cut on a sliding microtome, mounted on Super Frost plus slides (Fisher Scientific, Pittsburgh, PA), dehydrated under vacuum and then frozen at -80°C until processing for TUNEL assay.

TUNEL assay

For the *in situ* analysis of DNA fragmentation, the TUNEL staining was performed using a commercial kit (ApopTag Kit-S7100, Chemicon, Temecula, CA). The manufacturer's instructions were followed. Briefly, frozen sections were post-fixed with 1%

paraformaldehyde for 15 min followed by a cold solution of ethanol: acetic acid (2:1) for 5 min. Then the slides were washed 2 times with 50 mM phosphate buffer saline (PBS), immersed in 0.5% triton for 15 min, washed 2 times, digested with proteinase K (20 µg/ml) for 15 min and endogenous peroxidase was quenched with 3% H₂O₂ for 20 min. After 2 washings, the sections were incubated with TdT enzyme at 37°C for 90 min. This was followed by incubation with anti-digoxigenin-peroxidase for 60 min at room temperature, and color development with H₂O₂-3, 3' diaminobenzidine tetrahydrochloride (DAB, Dojindo Laboratories, Kumamoto, Japan) for 20 min, followed by 3 washes in milli-Q water. Sections were dehydrated in butanol followed by xylene and then mounted on Superfrost Plus slides (Fischer, Santa Clara, CA) under glass with DPX. Some of the slides were counterstained with 0.5% methyl green and coverslipped. Specimens of post-weaning mammary gland tissue from rats were provided by Chemicon to use as positive control tissue. Extensive apoptosis occurs 3-5 days after weaning of rat pups and post-weaning mammary tissue presents typical chromatin fragmentation labeled with TUNEL. Omission of TdT enzyme from the incubation buffers during processing constituted the technical negative control. Examination of other areas outside of the dorsal raphe constituted the anatomical negative control. Exposure of sections to DNase (700 U/ml) for 1-20 minutes prior to processing through the TUNEL assay constituted a positive control. The region of the dorsal raphe was analyzed and the morphometrical evaluation was made at a magnification of 1,000X with careful registration of the morphological features.

Quantitative Tissue Analysis

Sections were morphologically matched between animals using anatomical reference points and a rhesus monkey brain atlas (24). The Marianas stereological workstation with Slidebook 4.2 was used for analysis. Eight sections were examined through the rostro-caudal extent of the dorsal raphe with a 250µm interval between the sections. A montage of the dorsal raphe area at each level was built by the workstation. For cell counting, the dorsal raphe area was outlined on the montage, defined and measured (µ² total area). Then, TUNEL-positive cells were identified and counted by hand. Type I and type II staining were included. Complete dark staining of the nucleus is called type I, and peripheral staining in the perinuclear area is called type II (25, 26).

The number of TUNEL-positive neurons in the designated area of the eight sections was summed, generating one value of total TUNEL-positive cells for each animal. The individual sums were averaged to obtain the group mean. Slidebook 4.2 provides the square microns of the outlined area. The thickness of each section (25 µm × 8 sections = 200 µm) in microns was used as the length of the region. The average area of the region for each animal (µ² outlined by Slidebook 4.2) was multiplied by the length to obtain the volume of the analyzed region (µ³/10⁹ = mm³). The total number of TUNEL-positive neurons in each animal was divided by the volume of the area examined to obtain the number of TUNEL-positive neurons per cubic millimeter for each animal. The individual numbers of TUNEL-positive neurons /mm³ were then averaged to obtain the group means. Differences in cell numbers between the groups were determined with a Mann-Whitney nonparametric test using Prism Statistical software (Graph-Pad Software, Inc., San Diego, CA, USA) and p < 0.05 was considered statistically significant.

Double immunostaining TUNEL + tryptophan hydroxylase (TPH)

Tissue from an ovariectomized animal with a high number of TUNEL-positive neurons was used to perform the double immunostaining for TUNEL and TPH in the dorsal raphe nucleus. The TUNEL staining was executed as described above, using the ApopTag Kit-S7100 commercial kit. After development of the TUNEL staining in DAB, the sections were washed in Tris 0.05M for 5 minutes, washed in 0.02 KPBS (4 times at 15 min each) and post-fixed in 4% paraformaldehyde for 90 minutes. Then, they were washed 4 times with 0.02 M KPBS (15 min each) and incubated with the following blocking solutions: normal goat serum (0.6% NGS, Vector Laboratories, Burlingame, CA) for 1 hour; bovine serum albumin (3% BSA, Sigma, St. Louis, MO) for 1 hour; avidin for 20 min and biotin for 20 min (Vector Laboratories, Burlingame, CA). Sections were incubated at 4°C for 24 h in a rabbit antibody to human TPH (Novus Biologicals, Littleton, CO) diluted 1/3000 in 0.6% NGS, 0.4% triton and KPBS. Sections were rinsed 4 times in KPBS buffer (15 min each), incubated in biotinylated goat anti-rabbit serum for 1 hour (Vector Laboratories, Burlingame, CA), washed 4 times in KPBS buffer (15 min each), incubated with ABC-alkaline phosphatase (ABC-AP) reagent (Vector Laboratories, Burlingame, CA) for 1 hour, washed 4 times in KPBS buffer (15 min each), incubated with blue AP substrate kit (Vector Laboratories, Burlingame, CA) for 35 minutes and washed 2 times in Tris 0.05M (5 min each). Then, a drop of a mounting medium (DAPI, SlowFade Gold, Molecular Probes) was applied to each section, followed by the coverslip. Photomicrographs of the sections were obtained immediately on a Leica DMLB microscope with a Leica DFC290 digital camera.

Hormone Assays

Assays for E and P were performed utilizing a Roche Diagnostics 2010 Elecsys assay instrument. Before these analyses, measurements of E and P on this platform were compared to traditional RIAs as reported previously (27).

Results

TUNEL staining in the dorsal raphe nucleus

Figure 1 illustrates TUNEL staining in the rat mammary gland (positive control tissue) and in the dorsal raphe nucleus. Panels A and B contain photomicrographs of the *rat mammary gland* in the absence (A) and presence (B) of methyl green counterstain. White arrows indicate TUNEL-positive cells in the rat mammary tissue. Panels C and D contain photomicrographs of the *dorsal raphe nucleus* in the absence (C) and presence (D) of methyl green counterstain. The TUNEL positive cells are scattered across the dorsal raphe so distant cells were placed in inserts to panels C and D. The DNA fragmentation detected in the dorsal raphe by the TUNEL assay occurs in two forms referred to as type I and type II (28-30). Complete dark staining of the nucleus, called type I, and peripheral staining in the perinuclear area, called type II, may reflect different stages of the DNA fragmentation process that starts in the periphery and moves inward (25). Alternatively, the perinuclear, type II staining could indicate DNA leakage from the nucleus (26). Single arrows indicate type I staining and double arrows indicate type II staining. Panel E contains a photomicrograph of a section that was pretreated with DNase for 10 minutes prior to TUNEL staining and methyl green counterstain. This treatment digested the DNA and more

nicks are available for labeling. Thus, there are more TUNEL-positive cells apparent in this small area. Panel F contains a photomicrograph of a counterstained section of the dorsal raphe in which the Tdt enzyme was omitted during the TUNEL assay. The TUNEL staining was abolished by omission of the Tdt enzyme in this negative control section.

In order to perform a stereological analysis of the dorsal raphe nucleus in which positive and negative staining could be segregated, it was necessary to omit the methyl green counterstain. Figure 2 contains montages of representative sections stained by TUNEL at three levels of the dorsal raphe nucleus from an ovariectomized placebo treated animal to illustrate the morphological features.

For visual comparison only, Figure 3 illustrates the TUNEL staining at the same level (level 5) from one representative animal of each treatment group. Visually, there was a robust decrease in the number of TUNEL-positive cells in the dorsal raphe nucleus from animals treated with E alone, P alone and E+P compared to the placebo treated group (OVX).

Eight levels of the dorsal raphe nucleus, which were 250 μ apart in a rostral to caudal direction from all 20 animals were processed for TUNEL staining (n=5 animals/group) and the results are illustrated in Figure 4. However, there was individual variation between monkeys hindering analysis of variance across all treatment groups. Nonetheless as illustrated in Figure 4A, comparison of individual groups with the OVX-placebo group using a Mann-Whitney nonparametric test confirmed that both P treatment and E+P treatment caused a significant decrease in the total number of TUNEL-positive cells in the 8 levels of dorsal raphe nucleus that were measured ($p=0.04$, both treatments). When the number of TUNEL-positive cells/cubic millimeter (mm^3) was computed, there was a significant reduction of TUNEL-positive cells after treatment with E+P ($p=0.04$), and a nearly significant reduction occurred with P-alone ($p=0.1$) as illustrated in Figure 4B. The total area that was measured did not differ between groups (Figure 4C).

Double immunostaining for TUNEL plus tryptophan hydroxylase (TPH)

To determine if serotonin neurons exhibited DNA fragmentation, sections from an OVX placebo-treated animal were double immunolabeled for TUNEL plus TPH. In this experiment, the TUNEL was developed with DAB yielding a nuclear or perinuclear brown reaction product, and TPH was developed with alkaline phosphatase and blue substrate yielding a blue cytoplasmic product. Figure 5 contains photomicrographs of serotonin neurons that exhibit DNA fragmentation as well as, TUNEL-negative serotonin neurons. Type I and type II TUNEL staining was observed in serotonin neurons. The double-labeled neurons with brown TUNEL staining and blue TPH staining are indicated with 2 different black arrows. Serotonin neurons with type I staining are indicated with black single arrows and serotonin neurons with type II staining are indicated with black double-headed arrows. Serotonin neurons, which lack detectable TUNEL staining, are indicated with green arrows. In addition, the TUNEL-positive serotonin neurons exhibit different degrees of cellular degradation. That is, some neurons are still relatively intact with dense TPH cytoplasmic staining whereas other neurons are degraded with little visible cytoplasmic TPH. TUNEL-positive neurons that appear severely degraded and disintegrated are indicated with red arrows. This leads to the speculation that a significant number of the TUNEL-positive cells

with little or no visible TPH staining may have been serotonergic, but degradation and disintegration were advanced and/or phagocytosis was underway at the time of euthanasia.

Hormone Concentrations

Serum estradiol concentrations (pg/ml±SEM) equaled 8.4±1.7, 78.6±7.1, 14.0±6.1, and 89.0±12.7 in the placebo, E, P and E+P treated groups, respectively (n=5/treatment; ANOVA p <0.0001). Serum progesterone concentrations (ng/ml±SEM) equaled 0.09±0.02, 0.14±0.02, 6.1±0.6 and 3.8±0.7 in the placebo, E, P and E+P treated groups, respectively (n=5/treatment; ANOVA p <0.0001). The estrogen concentrations are similar to levels observed in the early to mid-follicular phase and the progesterone concentrations are similar to levels observed in the mid-luteal phase. Estrogen is also present at these concentrations in the mid-luteal phase.

Discussion

Several methods are available to detect apoptosis or necrosis. The most labor intensive is electron microscopy, which can detect nuclear fragmentation. This procedure is difficult to apply to large areas and suffers from low numbers of total recorded events. DNA laddering can be detected in cell homogenates but morphological features are lost. Another well accepted method is the TUNEL assay. TUNEL (terminal deoxynucleotidyl transferase nick end labeling) is commonly employed to detect DNA condensation and fragmentation although it cannot distinguish between different kinds of cell death such as apoptosis or necrosis. Nonetheless, one is able to screen large areas of neurons for early and late stages of DNA fragmentation.

Using a model of surgical menopause in monkeys, this study showed that progesterone administered alone or in conjunction with estrogen, decreased the number of TUNEL-positive cells in the dorsal raphe nucleus, and that TUNEL staining co-localized in serotonin neurons in the dorsal raphe nucleus. This implies a protective role for ovarian steroids in serotonergic neurons in nonhuman primates that did not suffer any kind of experimental brain injury.

Apoptosis is a highly regulated cellular process, with an important role in the regulation of the number of cells during development and in the maintenance of tissue homeostasis in adult life (31). Apoptosis can proceed by a caspase-dependent pathway, or a caspase-independent pathway that culminates in the translocation of apoptosis inducing factor (AIF). The caspases are present in the cytoplasm in an inactive proform and require proteolytic processing to be activated. Then, they cleave cytoskeletal and nuclear proteins, which are essential for maintenance of cell structure, as well as enzymes related to metabolic and repair mechanisms (12). They also can cleave the anti-apoptotic protein Bcl-2, which results in a pro-apoptotic cleavage product (32).

Several studies have shown that the caspase-dependent pathway is activated following injury to neural tissue (33-35), but few studies have examined apoptosis and/or neuroprotective effects of steroids in the non-injured brain, such as would be the case in the majority of postmenopausal women. Other studies have shown that estrogen and progesterone increase

the expression of anti-apoptotic genes and/or down-regulate the pro-apoptotic ones (22, 36-39). In rodent models, administration of estrogen prior to, or coincident with, trauma or global ischemia decreased tissue damage (40, 41). Moreover, caspases were activated in the damaged tissue suggesting that the caspase-dependent pathway is involved in apoptosis resulting from ischemia (31, 42). In addition, estrogen treatment decreases caspase activation in the damaged area of the brain (43). In the neocortex of rats injected with gp120, a human glycoprotein, which may be responsible for the neuronal loss observed post-mortem in the brain of AIDS patients, estrogen reduced apoptosis by a mechanism involving activation of estrogen receptors (ER) (44).

We previously found that ovarian steroid treatment decreased the expression of a number of pivotal genes in the caspase-dependent and caspase-independent pathways in laser captured neurons from non-injured macaques (22). To determine if these changes in gene expression translated to changes in protein expression, we examined apoptosis-related proteins in a small block of the midbrain containing the dorsal raphe nucleus from monkeys that were ovariectomized and treated with placebo, estrogen or estrogen plus progesterone. We found that hormone treatment significantly decreased JNK1 and AIF expression, and decreased the translocation of AIF from the mitochondria to the nucleus (23), suggesting that in the absence of overt injury to the midbrain, ovarian steroids suppress the caspase-independent pathway in the dorsal raphe nucleus. This would encourage serotonin neuronal resilience and decrease cellular vulnerability to stresses associated with normal life such as disease-generated cytokines, free radicals or psychological trauma.

This study confirms the hypothesis that ovarian steroids protect serotonin neurons from DNA fragmentation, which may be an indication of neuroendangerment. Although there was a downward trend with estrogen treatment, progesterone appears to play a more important role than previously thought, in that progesterone alone and estrogen plus progesterone treatment significantly reduced TUNEL-positive cells. TUNEL staining could not distinguish between DNA fragmentation due to apoptosis or necrosis. However, apoptotic cells are phagocytosed by nearby resident cells, typically without generating an acute inflammatory response. Therefore, infiltration of neutrophils does not occur with apoptosis as it does with ischemic cellular necrosis. We did not observe neutrophil infiltration in the dorsal raphe suggesting that the DNA fragmentation is a result of apoptotic pathway activation.

There was individual variation within the treatment groups, which may have decreased with more animals, but the cost of nonhuman primates is prohibitive. We only treated with ovarian steroids for one month and this may also be a contributing factor. That is, longer treatment periods may have resulted in more consistent reduction of TUNEL staining. Likewise, these were Chinese rhesus macaques and they were not genotyped. Hence as with human populations, there could be genetic predisposition and/or other developmental variables that render a primate more or less sensitive to the actions of ovarian steroids on DNA fragmentation.

It may be questioned how E and P decrease DNA fragmentation after one month of treatment. That is, are they reversing a process that is underway? We speculate that DNA

repair is possible if the fragmentation process has not proceeded to an advanced stage. Indeed, some of the milder affected cells with minor type II staining could be rescued with hormone therapy. Future studies on DNA repair mechanisms are certainly indicated. Moreover, this question redirects attention to the issue of the length of time after ovariectomy before hormone therapy is initiated. Our animals were treated between 3 and 8 months after ovariectomy. After longer time periods, DNA fragmentation may be more advanced, neurons may die and rescue may become impossible. Moreover, there may be a difference between 3 and 8 months of ovariectomy, which is more profound than previously anticipated. Unfortunately, it is extremely unlikely to have 20 animals available for assignment that were ovariectomized at the same time, so we have to use a practical time frame.

Recent clinical studies have shown that hormone therapy administered during the perimenopausal period is beneficial for treatment of depressive and anxious symptoms associated with the onset of menopause (45, 46). However, the Women's Health Initiative found that hormone therapy initiated many years after menopause was harmful in neural systems that have a serotonergic component, such as integrative cognition (47). Moreover, Brinton and colleagues demonstrated that estrogen treatment of unhealthy cells is disastrous for the cell, whereas estrogen treatment of healthy cells is neuroprotective (48, 49). In light of the data from this study, it is attractive to speculate that hormone therapy administered long after menopause will act deleteriously on the advanced TUNEL- positive cells of the dorsal raphe nucleus and promote the death of serotonin neurons. Conversely, hormone therapy at perimenopause will act in the raphe with few TUNEL- positive cells and thus promote cellular resilience amongst the serotonin neuronal population. Extrapolating from this data it is reasonable to speculate that there would be loss of serotonin neurons with aging and accompanying diseases. Studies of serotonin cell number, serotonin function and serotonin sensitivity in animals and humans across the lifespan and at different times after ovariectomy would be very informative.

The neuroprotective role of progesterone has been studied in different experimental models such as traumatic brain injury, induced ischemia and spinal cord lesions (50). In rats, after traumatic brain injury, progesterone treatment reduces edema and increases the number of surviving neurons (51, 52). In mice, progesterone has a beneficial effect on motor ability and spatial memory performance after cerebral ischemia (53) and in a model of brain injury in rats, progesterone decreased apoptotic cell death by decreasing expression of the pro-apoptotic protein, caspase-3 (54). Progesterone significantly reduced the neuronal loss in hippocampus and in the caudate nucleus after global ischemia (55, 56). Moreover progesterone exhibits neuroprotective effects in several neurodegenerative conditions such as stroke, Alzheimer's disease and schizophrenia (50, 57).

Notably, allopregnanolone, a progesterone metabolite that acts at GABA-A receptors, exhibits anti-apoptotic effects and reduces functional deficits in rats after traumatic brain injury (54), suggesting that allopregnanolone could be the molecule through which progesterone induces its neuroprotective effects. In the absence of estrogen treatment, the expression of nuclear progesterin receptors (PR) would be reduced (58, 59). Therefore, in this study the decrease in TUNEL staining observed in the progesterone-only treatment group is

consistent with an action through allopregnanolone. In the estrogen+progesterone treatment group, nuclear PR would be induced by estrogen, and therefore progesterone could additionally work through PR to promote cellular resilience. Likewise, the decrease in TUNEL staining with estrogen+progesterone could be solely due to an action of allopregnanolone. The intracellular crosstalk between allopregnanolone, the GABA-A receptor and the apoptosis-related pathways needs further investigation.

TUNEL staining was restricted to the dorsal and median raphe nuclei in that it was not observed in other regions of the midbrain. As shown in our previous work, AIF immunostaining was confined to the serotonin-like neurons of the raphe and was not observed in other midbrain structures either (23). Thus, TUNEL staining and AIF staining were largely confined to serotonin neurons in the midbrain, which also express robust concentrations of NFkB (60). Altogether the data from the microarray studies, the protein studies and the TUNEL studies point to ovarian steroids decreasing activity of the caspase-independent pathway and preventing re-entry into the cell cycle. Upon steroid withdrawal, there is an increase in cyclins, and an increase in JNK1 and AIF translocation to the nucleus, which lead to DNA fragmentation. In addition, there is a robust pool of NFkB in serotonin neurons, and NFkB translocation to the nucleus increases upon steroid withdrawal. Each of these proteins transduces stress signals such as cytokine and toll receptor activation.

Altogether, ovarian steroids, by affecting the balance between apoptotic and anti-apoptotic genes, may protect serotonin neurons from cell death induced by several stimuli such as hypoxia, oxidative stress, neurotoxic agents or glucose deprivation (61). However, different mechanisms may be involved in normal and injured tissues. Comparison of our data with studies in injured brain requires consideration that DNA degradation depends on several interacting pathways and different insults (or their absence) can activate different signaling events. Consequently this may lead to different morphology for chromatin condensation and DNA fragmentation (62).

In summary, progesterone alone or in conjunction with estrogen, was neuroprotective in an animal model of surgical menopause by decreasing DNA fragmentation in serotonin neurons of the dorsal raphe nucleus. This action would increase the cellular resilience of serotonin neurons to normal life stresses and may prevent premature serotonin neuron death in women facing decades of life after menopause.

Acknowledgements

We are deeply grateful to the dedicated staff of the Division of Animal Resources including the staff of the Departments of Surgery and Pathology for their expertise and helpfulness in all aspects of monkey management. We especially thank Dr. Jessica Henderson for cutting the midbrain brain blocks and organizing the sections. We are indebted to the Endocrine Services Laboratory for radioimmunoassay of steroid hormones.

This research was supported by NIH-Grants MH 62677 to CLB, U54-HD18185, and RR00163 for Support of the Oregon National Primate Research Center.

Literature Cited

1. Simpkins JW, Yang SH, Wen Y, Singh M. Estrogens, progestins, menopause and neurodegeneration: basic and clinical studies. *Cell Mol Life Sci.* Feb; 2005 62(3):271–280. [PubMed: 15723163]
2. Singh M, Dykens JA, Simpkins JW. Novel mechanisms for estrogen-induced neuroprotection. *Exp Biol Med (Maywood).* May; 2006 231(5):514–521. [PubMed: 16636299]
3. Suzuki S, Brown CM, Wise PM. Mechanisms of neuroprotection by estrogen. *Endocrine.* Apr; 2006 29(2):209–215. [PubMed: 16785597]
4. Isacson O. On neuronal health. *Trends Neurosci.* Aug; 1993 16(8):306–308. [PubMed: 7691004]
5. Manji HK, Duman RS. Impairments of neuroplasticity and cellular resilience in severe mood disorders: implications for the development of novel therapeutics. *Psychopharmacol Bull.* 2001 Spring;35(2):5–49. [PubMed: 12397885]
6. Teeling JL, Perry VH. Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: underlying mechanisms. *Neuroscience.* 2009; 158:1062–1073. [PubMed: 18706982]
7. Tejani-Butt SM, Yang J, Pawlyk AC. Altered serotonin transporter sites in Alzheimer's disease raphe and hippocampus. *NeuroReport.* 1995; 6:1207–1210. [PubMed: 7662909]
8. Benninghoff J, Schmitt A, Mossner R, Kesch K-P. When cells become depressed: focus on neural stem cells in novel treatment strategies against depression. *J Neural Transm.* 2002; 109:947–962. [PubMed: 12111481]
9. McEwen BS. Stress and hippocampal plasticity. *Annu Rev Neurosci.* 1999; 22:105–122. [PubMed: 10202533]
10. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* Aug; 1972 26(4):239–257. [PubMed: 4561027]
11. Peter ME, Heufelder AE, Hengartner MO. Advances in apoptosis research. *Proc Natl Acad Sci USA.* Nov 25; 1997 94(24):12736–12737. [PubMed: 9398063]
12. Gorman AM, Orrenius S, Ceccatelli S. Apoptosis in neuronal cells: role of caspases. *Neuroreport.* Jul 13; 1998 9(10):R49–55. [PubMed: 9694191]
13. Krantic S, Mechawar N, Reix S, Quirion R. Apoptosis-inducing factor: a matter of neuron life and death. *Prog Neurobiol.* Feb; 2007 81(3):179–196. [PubMed: 17267093]
14. Fuchs E, Czeh B, Kole MH, Michaelis T, Lucassen PJ. Alterations of neuroplasticity in depression: the hippocampus and beyond. *Eur Neuropsychopharmacol.* Dec; 2004 14(Suppl 5):S481–490. [PubMed: 15550346]
15. Bethea CL, Centeno ML, Cameron JL. Neurobiology of stress-induced reproductive dysfunction in female macaques. *Mol Neurobiol.* Dec; 2008 38(3):199–230. [PubMed: 18931961]
16. Gundlah C, Lu NZ, Mirkes SJ, Bethea CL. Estrogen receptor beta (ER β) mRNA and protein in serotonin neurons of macaques. *Molecular Brain Research.* 2001; 91:14–22. [PubMed: 11457488]
17. Bethea CL. Colocalization of progestin receptors with serotonin in raphe neurons of macaque. *Neuroendocrinology.* 1993; 57:1–6. [PubMed: 8479605]
18. Bethea CL, Lu NZ, Gundlah C, Streicher JM. Diverse actions of ovarian steroids in the serotonin neural system. *Frontiers in Neuroendocrinology.* 2002; 23:41–100. [PubMed: 11906203]
19. Lu NZ, Bethea CL. Ovarian steroid regulation of 5HT1A receptor binding and G protein activation in female monkeys. *Neuropsychopharmacology.* 2002; 27:12–24. [PubMed: 12062903]
20. Lu NZ, Eshleman AJ, Janowsky A, Bethea CL. Ovarian steroid regulation of serotonin reuptake transporter (SERT) binding, distribution and function in female macaques. *Molecular Psychiatry.* 2003; 8:353–360. [PubMed: 12660809]
21. Reddy AP, Bethea CL. Preliminary array analysis reveals novel genes regulated by ovarian steroids in the monkey raphe region. *Psychopharmacology (Berl).* Jun; 2005 180(1):125–140. [PubMed: 15731897]
22. Bethea C, Reddy A. Effect of ovarian hormones on survival genes in laser captured serotonin neurons from macaques. *J Neurochem.* 2008; 105:1129–1143. [PubMed: 18182058]

23. Tokuyama Y, Reddy A, Bethea C. Neuroprotective actions of ovarian hormones without insult in the raphe region of rhesus macaques. *Neuroscience*. 2008; 154:720–731. [PubMed: 18486349]
24. Paxinos, G.; Huang, X-F.; Toga, AW. *The Rhesus Monkey Brain in Stereotaxic Coordinates*. Academic Press; New York: 2000.
25. Chang GQ, Hao Y, Wong F. Apoptosis: final common pathway of photoreceptor death in rd, rds, and rhodopsin mutant mice. *Neuron*. Oct; 1993 11(4):595–605. [PubMed: 8398150]
26. Conti AC, Raghupathi R, Trojanowski JQ, McIntosh TK. Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period. *J Neurosci*. Aug 1; 1998 18(15):5663–5672. [PubMed: 9671657]
27. Bethea CL, Streicher JM, Mirkes SJ, Sanchez RL, Reddy AP, Cameron JL. Serotonin-related gene expression in female monkeys with individual sensitivity to stress. *Neuroscience*. 2005; 132(1): 151–166. [PubMed: 15780474]
28. Rink A, Fung KM, Trojanowski JQ, Lee VM, Neugebauer E, McIntosh TK. Evidence of apoptotic cell death after experimental traumatic brain injury in the rat. *Am J Pathol*. Dec; 1995 147(6): 1575–1583. [PubMed: 7495282]
29. Piantadosi CA, Zhang J, Levin ED, Folz RJ, Schmechel DE. Apoptosis and delayed neuronal damage after carbon monoxide poisoning in the rat. *Exp Neurol*. Sep; 1997 147(1):103–114. [PubMed: 9294407]
30. Wei L, Ying DJ, Cui L, Langsdorf J, Yu SP. Necrosis, apoptosis and hybrid death in the cortex and thalamus after barrel cortex ischemia in rats. *Brain Res*. Oct 1; 2004 1022(1-2):54–61. [PubMed: 15353213]
31. Springer JE. Apoptotic cell death following traumatic injury to the central nervous system. *J Biochem Mol Biol*. Jan 31; 2002 35(1):94–105. [PubMed: 16248974]
32. Cheng EH, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, et al. Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science*. Dec 12; 1997 278(5345):1966–1968. [PubMed: 9395403]
33. Clark RS, Kochanek PM, Watkins SC, Chen M, Dixon CE, Seidberg NA, et al. Caspase-3 mediated neuronal death after traumatic brain injury in rats. *J Neurochem*. Feb; 2000 74(2):740–753. [PubMed: 10646526]
34. Citron BA, Arnold PM, Sebastian C, Qin F, Malladi S, Ameenuddin S, et al. Rapid upregulation of caspase-3 in rat spinal cord after injury: mRNA, protein, and cellular localization correlates with apoptotic cell death. *Exp Neurol*. Dec; 2000 166(2):213–226. [PubMed: 11085887]
35. Eldadah BA, Faden AI. Caspase pathways, neuronal apoptosis, and CNS injury. *J Neurotrauma*. Oct; 2000 17(10):811–829. [PubMed: 11063050]
36. Alkayed NJ, Goto S, Sugo N, Joh HD, Klaus J, Crain BJ, et al. Estrogen and Bcl-2: gene induction and effect of transgene in experimental stroke. *J Neurosci*. Oct 1; 2001 21(19):7543–7550. [PubMed: 11567044]
37. Garcia-Segura LM, Cardona-Gomez P, Naftolin F, Chowen JA. Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroreport*. Mar 9; 1998 9(4):593–597. [PubMed: 9559922]
38. Yao M, Nguyen TV, Pike CJ. Estrogen regulates Bcl-w and Bim expression: role in protection against beta-amyloid peptide-induced neuronal death. *J Neurosci*. Feb 7; 2007 27(6):1422–1433. [PubMed: 17287517]
39. Nilsen J, Brinton RB. Impact of progestins on estrogen-induced neuroprotection: Synergy by progesterone and 19-norprogesterone and antagonism by medroxyprogesterone acetate. *Endocrinology*. 2002; 143(1):205–212. [PubMed: 11751611]
40. Hoffman GE, Merchenthaler I, Zup SL. Neuroprotection by ovarian hormones in animal models of neurological disease. *Endocrine*. Apr; 2006 29(2):217–231. [PubMed: 16785598]
41. McCullough LD, Alkayed NJ, Traystman RJ, Williams MJ, Hurn PD. Postischemic estrogen reduces hypoperfusion and secondary ischemia after experimental stroke. *Stroke*. Mar; 2001 32(3): 796–802. [PubMed: 11239204]
42. Faubel S, Edelstein CL. Caspases as drug targets in ischemic organ injury. *Curr Drug Targets Immune Endocr Metabol Disord*. Sep; 2005 5(3):269–287. [PubMed: 16178788]
43. Jover-Mengual T, Zukin RS, Etgen AM. MAPK signaling is critical to estradiol protection of CA1 neurons in global ischemia. *Endocrinology*. Mar; 2007 148(3):1131–1143. [PubMed: 17138646]

44. Corasaniti MT, Amantea D, Russo R, Piccirilli S, Leta A, Corazzari M, et al. 17beta-estradiol reduces neuronal apoptosis induced by HIV-1 gp120 in the neocortex of rat. *Neurotoxicology*. Oct; 2005 26(5):893–903. [PubMed: 15922453]
45. Schmidt PJ, Nieman L, Danaceau MA, Tobin MB, Roca CA, Murphy JH, et al. Estrogen replacement in perimenopause-related depression: a preliminary report. *Am J Obstet Gynecol*. 2000; 183:414–420. [PubMed: 10942479]
46. Frey BN, Lord C, Soares CN. Depression during menopausal transition: a review of treatment strategies and pathophysiological correlates. *Menopause Int*. Sep; 2008 14(3):123–128. [PubMed: 18714078]
47. Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, et al. Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. *JAMA*. Jun 23; 2004 291(24):2959–2968. [PubMed: 15213207]
48. Zhao L, O'Neill K, Brinton R Diaz. Selective estrogen receptor modulators (SERMs) for the brain: current status and remaining challenges for developing NeuroSERMs. *Brain Res Brain Res Rev*. Nov; 2005 49(3):472–493. [PubMed: 16269315]
49. Brinton RD. Investigative models for determining hormone therapy-induced outcomes in brain: evidence in support of a healthy cell bias of estrogen action. *Ann N Y Acad Sci*. Jun.2005 1052:57–74. [PubMed: 16024751]
50. Schumacher M, Guennoun R, Stein DG, De Nicola AF. Progesterone: therapeutic opportunities for neuroprotection and myelin repair. *Pharmacol Ther*. Oct; 2007 116(1):77–106. [PubMed: 17659348]
51. Galani R, Hoffman SW, Stein DG. Effects of the duration of progesterone treatment on the resolution of cerebral edema induced by cortical contusions in rats. *Restor Neurol Neurosci*. 2001; 18(4):161–166. [PubMed: 11847439]
52. Cutler SM, VanLandingham JW, Murphy AZ, Stein DG. Slow-release and injected progesterone treatments enhance acute recovery after traumatic brain injury. *Pharmacol Biochem Behav*. Jul; 2006 84(3):420–428. [PubMed: 16870241]
53. Gibson CL, Murphy SP. Progesterone enhances functional recovery after middle cerebral artery occlusion in male mice. *J Cereb Blood Flow Metab*. Jul; 2004 24(7):805–813. [PubMed: 15241189]
54. Djebaili M, Guo Q, Pettus EH, Hoffman SW, Stein DG. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. *J Neurotrauma*. Jan; 2005 22(1):106–118. [PubMed: 15665606]
55. Gonzalez-Vidal MD, Cervera-Gaviria M, Ruelas R, Escobar A, Morali G, Cervantes M. Progesterone: protective effects on the cat hippocampal neuronal damage due to acute global cerebral ischemia. *Arch Med Res*. 1998 Summer;29(2):117–124. [PubMed: 9650325]
56. Cervantes M, Gonzalez-Vidal MD, Ruelas R, Escobar A, Morali G. Neuroprotective effects of progesterone on damage elicited by acute global cerebral ischemia in neurons of the caudate nucleus. *Arch Med Res*. Jan-Feb;2002 33(1):6–14. [PubMed: 11825624]
57. Garcia-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by estradiol. *Prog Neurobiol*. Jan; 2001 63(1):29–60. [PubMed: 11040417]
58. Bethea CL. Regulation of progestin receptors in raphe neurons of steroid-treated monkeys. *Neuroendocrinology*. 1994; 60:50–61. [PubMed: 8090282]
59. Shughrue PJ, Lane MV, Merchenthaler I. Regulation of progesterone receptor messenger ribonucleic acid in the rat medial preoptic nucleus by estrogenic and antiestrogenic compounds: an in situ hybridization study. *Endocrinology*. 1997; 138:5476–5484. [PubMed: 9389534]
60. Bethea CL, Reddy AP, Smith LJ. Nuclear factor kappa B in the dorsal raphe of macaques: an anatomical link for steroids, cytokines and serotonin. *J Psychiatry Neurosci*. Mar; 2006 31(2):105–114. [PubMed: 16575426]
61. Maggi A, Ciana P, Belcredito S, Vegeto E. Estrogens in the nervous system: mechanisms and nonreproductive functions. *Annu Rev Physiol*. 2004; 66:291–313. [PubMed: 14977405]
62. Tatton WG, Chalmers-Redman R, Brown D, Tatton N. Apoptosis in Parkinson's disease: signals for neuronal degradation. *Ann Neurol*. 2003; 53(Suppl 3):S61–70. discussion S70-62. [PubMed: 12666099]

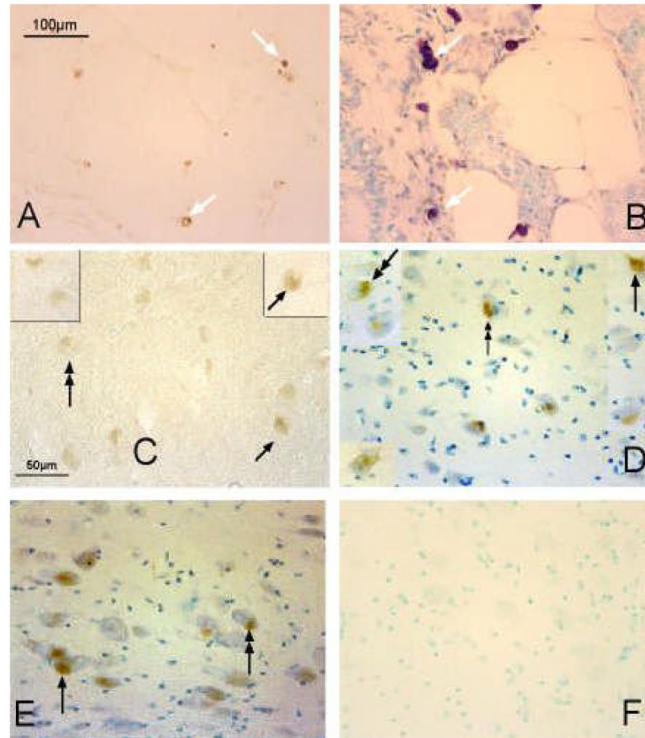


Figure 1.

Illustration of DNA fragmentation and TUNEL staining in the post-weaning rat-mammary gland and in the dorsal raphe nucleus of rhesus monkeys. There are 2 staining patterns observed which have been previously reported in other models and referred to as type I and type II. Complete dark staining of the nucleus, type I, and peripheral staining in the perinuclear area, type II, may reflect different stages of the DNA fragmentation process that starts in the periphery and moves inward. Alternatively, the perinuclear, type II staining could indicate DNA leakage from the nucleus. White arrows indicate TUNEL positive cells in the rat mammary gland. Single-head arrows indicate Type I staining and double-head arrows indicate Type II staining in the dorsal raphe nucleus. The magnification of Panel A applies to Panel B. The magnification of Panel C also applies to Panels D, E and F.

Panel A - TUNEL staining of the mammary gland without counterstain.

Panel B - TUNEL staining of the mammary gland with methyl green counterstain.

Panel C - TUNEL staining in the dorsal raphe nucleus without counterstain.

Panel D — TUNEL staining of the dorsal raphe nucleus with methyl green counterstain.

Panel E- TUNEL staining of the dorsal raphe nucleus after exposure of the section to DNase for 10 minutes prior to TUNEL assay. There is an increase in the number of cells exhibiting different degrees of DNA fragmentation.

Panel F — negative control section of the dorsal raphe generated by omission of the Tdt reagent in the TUNEL assay. There was no apparent DNA fragmentation detected.

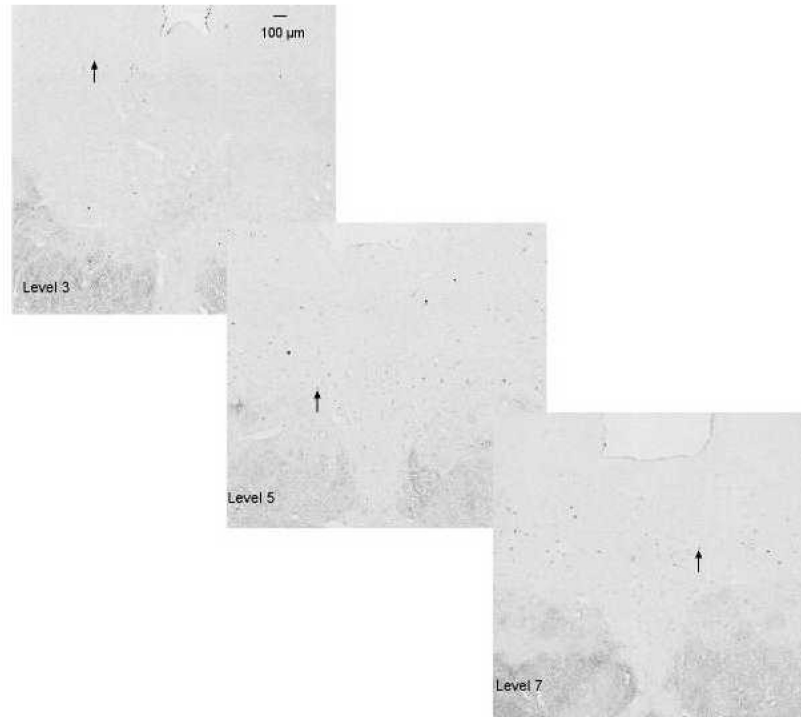


Figure 2. Illustration of the anatomical area where the TUNEL staining was counted. Photomicrographs of the montages of 3 levels (out of a total of 8 levels analyzed) of the dorsal raphe nucleus from an ovariectomized monkey treated with placebo that manifest robust TUNEL staining. The montage was built automatically by Slidebook 4.2 and the stereology microscope. Arrows indicate TUNEL positive cells.

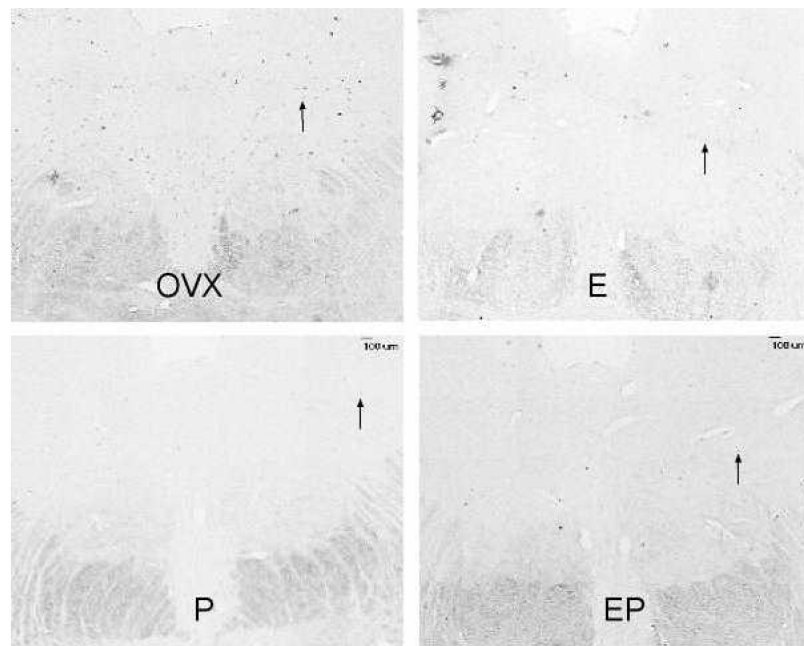


Figure 3. Comparison of TUNEL staining at the same level (level 5) of the dorsal raphe nucleus from a representative animal in each treatment group to visually illustrate the marked differences in the number of TUNEL positive cells. The animals were ovariectomized and treated with placebo (OVX), estradiol (E), progesterone (P) or estradiol plus progesterone (EP) for 28 days. Arrows indicate TUNEL positive cells.

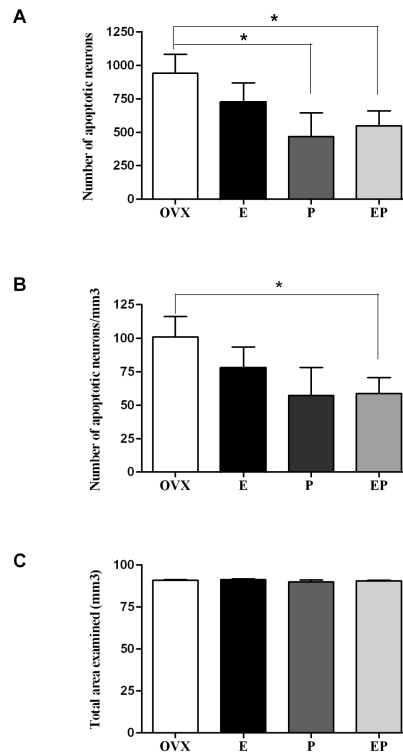


Figure 4.

Histograms illustrating the results of stereological analysis of the number of TUNEL-positive neurons in the dorsal raphe nucleus of rhesus macaques treated for 28 days with placebo (OVX), estradiol (E), progesterone (P) or estradiol plus progesterone (EP). TUNEL stained neurons were counted on the montage within a defined area (μ^2).

(A) Average (\pm SEM) of the total number of TUNEL-positive cells in 8 levels of the dorsal raphe nucleus in each treatment group (n=5 animals/group). There was a significant decrease in the total number of TUNEL positive neurons in the P and EP- treated groups compared with the OVX group ($p=0.04$ for P versus Ovx and $p=0.04$ for EP versus Ovx, Mann-Whitney nonparametric test).

(B) Average (\pm SEM) of the TUNEL-positive neurons per cubic millimeter (mm^3) of eight levels of the dorsal raphe nucleus in each treatment group (n=5 animals/group). There was a significant decrease in TUNEL-positive cells/ mm^3 in the E+P- treated group ($p=0.04$, Mann-Whitney nonparametric test), while the P group was nearly significantly different ($p=0.1$, Mann-Whitney nonparametric test).

(C) Comparison of the total volume of the area measured in each treatment group showed no difference.

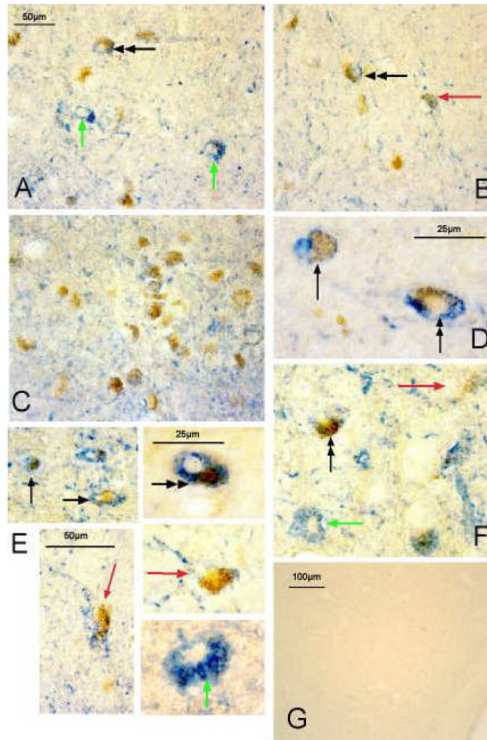


Figure 5.

Illustration of colocalization of TUNEL staining and tryptophan hydroxylase (TPH) in neurons of the dorsal raphe nucleus. Photomicrographs are from the dorsal raphe nucleus of an OVX monkey following double-immunohistochemistry for TPH (blue, cytoplasmic) and TUNEL (brown, nuclear, perinuclear). Single arrows indicate double-labeled neurons with type I TUNEL staining. Double-headed arrows indicate double-labeled neurons with type II TUNEL staining. Green arrows indicate neurons that are stained only for TPH. Red arrows indicate TUNEL-positive neurons that are in advanced stages of disintegration.

- A.** Photomicrograph of a region of the dorsal raphe containing 2, or possibly 3, serotonin neurons with no TUNEL staining (green arrows and adjacent cell). One cell is present with TUNEL staining in the perinuclear area and TPH staining in the cytoplasm (double arrow). Other TUNEL positive cells are apparent that are either not serotonergic or degradation has proceeded to the point that TPH is no longer present. The scale bar also applies to panels B, C and the upper left picture in panel E.
- B.** Photomicrograph of a region of the dorsal raphe containing TUNEL-positive cells at different stages of disintegration. The double-headed arrow highlights a TUNEL-positive cell with TPH-positive cytoplasm remaining. The red arrow highlights a TUNEL-positive cell in an advanced stage of degradation with little TPH-positive cytoplasm remaining. Other TUNEL positive cells are evident that are either not serotonergic or degradation has proceeded to the point that TPH is no longer present.

- C.** Photomicrograph of a region of the dorsal raphe from an ovariectomized macaque treated with placebo that exhibited very high numbers of TUNEL positive cells in the stereological analysis. There are few remnants of TPH-positive cytoplasm remaining in this cluster of dying cells.
- D.** Photomicrograph of two neurons double-labeled for TUNEL and TPH. The left neuron exhibits type I TUNEL staining (single arrow), which is predominantly nuclear, whereas the right neuron exhibits type II staining (double arrow) in which the DNA fragmentation is perinuclear.
- E.** Multi-panel with photomicrographs of TPH-positive neurons with different degrees of DNA fragmentation, and one TPH-positive neurons with no apparent TUNEL staining (bottom right, green arrow). Single arrows indicate serotonin neurons with type I TUNEL staining and double-headed arrows indicate serotonin neurons with type II TUNEL staining. The red arrows indicate serotonin neurons with TUNEL staining that is in an advanced stage of degradation as indicated by the sparse TPH staining.
- F.** Photomicrograph of a region of the dorsal raphe that contains a neuron that is double labeled for TUNEL and TPH (double arrow), a serotonin neuron with no apparent TUNEL stain (green arrow), and a TUNEL—positive neuron in an advanced stage of degradation (red arrow).
- G.** Photomicrograph of the periaqueductal gray region of the same section as panel C. There was no apparent TUNEL staining in this region. TUNEL staining was absent or rare in other adjacent neural structures of the midbrain suggesting that serotonin neurons are particularly vulnerable to steroid withdrawal.